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Chromosomes of *Pseudapanteles dignus* (Muesebeck, 1938) and a review of known karyotypes of the subfamily Microgastrinae (Hymenoptera, Braconidae)

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Abstract

The karyotype of *Pseudapanteles dignus* (Muesebeck, 1938), an important parasitoid of a serious tomato pest *Phthorimaea* (= *Tuta*) *absoluta* Meyrick, 1917 (Lepidoptera, Gelechiidae), in the Neotropics and adjacent regions, was studied for the first time using morphometric analysis and several techniques of differential chromosome staining, i.e., C-banding and staining with base-specific fluorochromes, together with fluorescence *in situ* hybridization (FISH) with an 18S rDNA probe. We found n = 7 and 2n = 14 in *P. dignus*, with seven metacentric chromosomes of similar size in the haploid set. C-banding revealed various C-positive bands, either centromeric or interstitial, on most chromosomes. Both AT-specific and GC-specific fluorochromes, 4'6-diamidino-2-phenylindole (DAPI) and chromomycin A_3 (CMA_3) respectively, showed uniform staining of chromosomes. FISH visualized a single subterminal rDNA site on a medium-sized metacentric. A brief review of known chromosome sets of the subfamily Microgastrinae (Braconidae) is given; certain features of karyotype evolution of this group are discussed.

Keywords

Base-specific fluorochromes, Braconidae, C-banding, chromosomes, fluorescence *in situ* hybridization, karyotypes, Microgastrinae, parasitoids

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Introduction

Parasitoid Hymenoptera are one of the most species-rich, taxonomically complicated and economically important groups of insects (Bebber et al. 2014; Forbes et al. 2018). In particular, the family Braconidae, with its high morphological and ecological diversity, contains more than 20,000 described species (Huber 2017). Moreover, Microgastrinae represent the second most speciose subfamily of Braconidae, which exceeds 3,000 described species, and up to 43,000 awaiting description, especially in the tropics (Rodriguez et al. 2013; Fernandez-Triana et al. 2020). Nevertheless, karyotypes of just a few members of this group are known so far, with only chromosomes of *Cotesia congregata* (Say, 1836) studied using differential staining (Belle et al. 2002; Gokhman 2009). We have examined the karyotype of another species from this subfamily, *Pseudapanteles dignus* (Muesebeck, 1938), an important solitary larval endoparasitoid of a serious worldwide tomato pest *Phthorimaea* (= *Tuta*) *absoluta* Meyrick, 1917 (Lepidoptera, Gelechiidae) in the Neotropics and adjacent regions (Fernandez-Triana et al. 2014), using several techniques of differential chromosome staining. The results of this work are given below. Several biological and ecological studies have shown that *P. dignus* can potentially control *P. absoluta*, either under natural conditions or by augmentative releases in tomato fields (Salas Gervassio et al. 2019; D´Auro et al. 2021; Vallina et al. 2022). Knowledge of genetic aspects of the parasitoid life history can therefore contribute to quality mass production of biocontrol agents, and consequently, to optimization of pest control (Lommen et al. 2017). In addition to the chromosomal study of *P. dignus*, we briefly review the current state of knowledge of karyotypic diversity of Microgastrinae.

Materials and methods

Origin of the material studied

The laboratory stock of *P. dignus* maintained at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE, CONICET and UNLP, La Plata, Argentina) originates from insects reared from cocoons of this parasitoid. These cocoons, containing pupae of *P. dignus*, were collected on tomato leaves infested with *P. absoluta* near La Plata (see Luna et al. 2007). This endoparasitoid species attacks second to fourth larval instars of gelechiid moths, particularly *P. absoluta*, depositing up to eight eggs per host during oviposition (D'Auro et al. 2021). However, only a single *P. dignus* larva survives to the third instar. At this stage, the parasitoid larva emerges from the dying host and pupates, typically spinning a silk cocoon (Luna et al. 2007). The preimaginal period lasts about 21 d; adults live ≥ 23 d in presence of the host (Vallina et al. 2022). To prepare the specimens for the chromosomal study, twenty cohorts of *P. dignus* were initiated by exposing a two-day-old, fertilized female to twenty larvae of *P. absoluta* inside leaf mines. The larvae were then kept in one-liter plastic containers and fed with 50% honey syrup

ad libitum. Cohorts were generated sequentially to synchronize the rearing process and to obtain material for dissections at the correct developmental stage. All cultures were maintained at 25 °C and 60 to 75% humidity, with a 14 h light: 10 h dark photoperiod in a walk-in environmental chamber. Voucher specimens from this study are deposited at CEPAVE (La Plata, Argentina).

Preparation and staining of chromosomes

Chromosomal preparations were obtained from cerebral ganglia of parasitoid prepupae generally following the protocol developed by Imai et al. (1988) with certain modifications (see, e.g., Gokhman et al. 2019). Ganglia were extracted from insects dissected in 0.5% hypotonic sodium citrate dihydrate solution containing 0.005% colchicine. The extracted ganglia were then transferred to fresh hypotonic solution and incubated for 30 min at room temperature. The material was transferred onto a pre-cleaned microscope slide using a Pasteur pipette and then gently flushed with Fixative I (glacial acetic acid: absolute ethanol: distilled water 3:3:4). The tissues were disrupted using dissecting needles in an additional drop of Fixative I. A drop of Fixative II (glacial acetic acid: absolute ethanol 1:1) was applied to the center of the area, and the more aqueous phase was blotted off the edges of the slide. The slides were dried for approximately 30 min and stored at room temperature.

For routine staining, chromosome preparations were stained overnight using a freshly prepared 3% Giemsa solution (Merck KGaA, Darmstadt, Germany). C-banding and sequential staining with AT-specific 4',6-diamidino-2-phenylindole (DAPI; Fluka BioChemika, Sigma Aldrich Production GmbH, Buchs, Switzerland) and GC-specific chromomycin A_3 (CMA_3; Fluka BioChemika) were carried out following Poggio et al. (2011). For C-banding, the pre-treated slides were stained with DAPI to improve the resolution of C-bands (Barros e Silva and Guerra 2010; Poggio et al. 2011).

Unlabeled 18S ribosomal DNA (rDNA) probe was generated by polymerase chain reaction (PCR) using universal arthropod primers: forward 5'-CCTGAGAAACGGCTACCACATC-3' and reverse 5'-GAGTCTCGTTCGTTATCGGA-3' (Whiting 2002). Total genomic DNA of *Dysdercus albofasciatus* Berg, 1878 (Hemiptera, Pyrrhocoridae), obtained by standard phenol-chloroform-isoamyl alcohol extraction, was used as a template. PCR was performed following the procedure described by Fuková et al. (2005) and Bressa et al. (2009). The PCR product displayed a single band of approximately 1,000 bp on a 1% agarose gel. The band was cut out from the gel, and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The 18S rDNA fragment was re-amplified by PCR and subsequently labeled with biotin-14-dUTP by nick translation using a BioNick Labeling System (Invitrogen, Life Technologies Inc., San Diego, CA, USA). FISH with biotinylated 18S rDNA probe was performed following the procedure developed by Sahara et al. (1999) with several modifications described by Fuková et al. (2005) and Bressa et al. (2009).

Image acquisition and analysis

Metaphase plates of *P. dignus* were examined and photographed with an optical microscope Zeiss Axioskop 40 FL fitted with a digital color camera Axiocam 208 (Carl Zeiss, Germany) as well as an epifluorescence microscope Leica DMLB fitted with a digital camera Leica DFC350 FX CCD (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK) respectively. To produce illustrations, the resulting images were processed with the image processing programs ZEN version 3.0 (blue edition), Leica IM50 version 4.0, Adobe Photoshop CC version 14.0, and GIMP version 2.10. Black-and-white images of chromosomes were captured separately for each fluorescent dye. Images were pseudocolorized (light blue, green, and red for DAPI, CMA_3, and Cy3, respectively) and processed with Adobe Photoshop CC version 14.0. KaryoType version 2.0 software (Altınordu et al. 2016) was also used for taking measurements from ten haploid metaphase plates of *P. dignus*. The chromosomes were classified following guidelines provided by Levan et al. (1964). All studies were conducted at CEPAVE (La Plata, Argentina), IEGEBA/DEGE of FCEyN of Universidad de Buenos Aires (Ciudad Autónoma de Buenos Aires, Argentina), and the Botanical Garden of Moscow State University (Moscow, Russia).

Results

The haploid karyotype of *P. dignus* contains seven metacentric chromosomes, which exhibit a gradual decrease in size (n = 7; Fig. 1A, B, Table 1). In prometaphase chromosomes, pericentromeric and interstitial heterochromatic segments are visible. In addition, a distinct secondary constriction is visible on a medium-sized chromosome (Fig. 1B). The diploid chromosome set of *P. dignus* consists of seven pairs of similar metacentric chromosomes (Fig. 1C; 2n = 14). Chromosome relative lengths of the

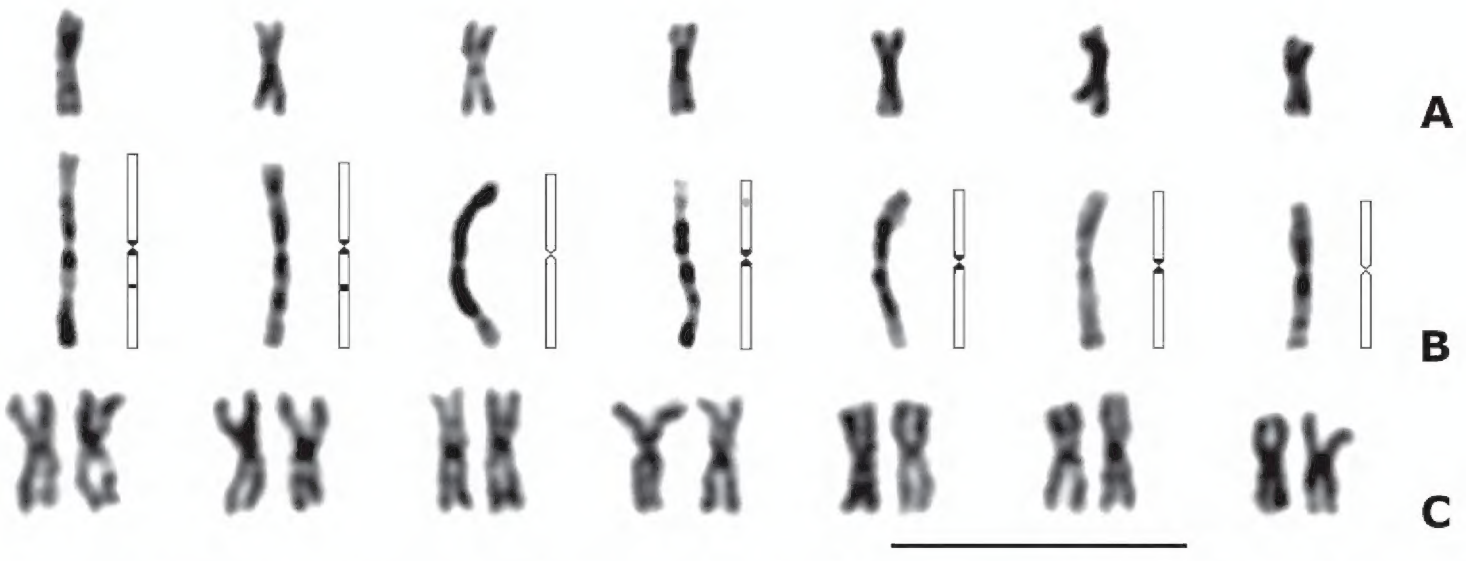

Figure 1. Karyograms of Giemsa-stained chromosomes of *P. dignus* **A** haploid, metaphase **B** haploid, prometaphase **C** diploid, metaphase. For **B** idiogram for each chromosome demonstrating heterochromatin distribution and position of the secondary constriction in black and grey respectively, is also shown. Scale bar: 10 μm.

Table 1. Relative lengths (RLs) and centromeric indices (CIs) of chromosomes of *P. dignus* (mean ± SD).

Chr. no.	RL, per cent	CI, per cent
1	16.15 ± 0.44	48.62 ± 0.86
2	15.42 ± 0.36	47.91 ± 2.68
3	14.67 ± 0.40	46.13 ± 2.74
4	14.35 ± 0.38	47.60 ± 1.71
5	13.87 ± 0.35	47.41 ± 2.03
6	13.19 ± 0.43	47.07 ± 2.38
7	12.35 ± 0.46	46.39 ± 2.43

haploid set (RLs) range from 16.15 ± 0.44 per cent for the longest chromosome to 12.35 ± 0.46 per cent for the smallest one (Table 1). Despite some difference in RLs between the longest and the shortest chromosome, the karyotype of *P. dignus* is fairly homogeneous in chromosome morphology and size, suggesting that this species possesses a highly symmetrical karyotype (Stebbins 1950).

C-banding reveals different patterns in the amount and location of constitutive heterochromatin on the chromosomes of *P. dignus*. Specifically, three pairs of chromosomes in the diploid set exhibit only centromeric C-positive bands. These bands are brighter and more conspicuous on chromosomes of two of the pairs than on the third one. On chromosomes of the two other pairs, strong C-positive centromeric bands are accompanied by small interstitial ones. No C-bands are detected on the remaining chromosomes (Fig. 2A, B). All mitotic chromosomes show relatively uniform fluorochrome staining with both DAPI and CMA_3 (Fig. 3).

In the diploid karyotype of *P. dignus*, FISH with an 18S rDNA probe reveals a single subterminal rDNA cluster on a pair of medium-sized metacentric chromosomes (Fig. 4). The location of the rDNA cluster apparently co-localizes with the secondary constriction observed on a specific medium-sized chromosome of this species (Fig. 1B).

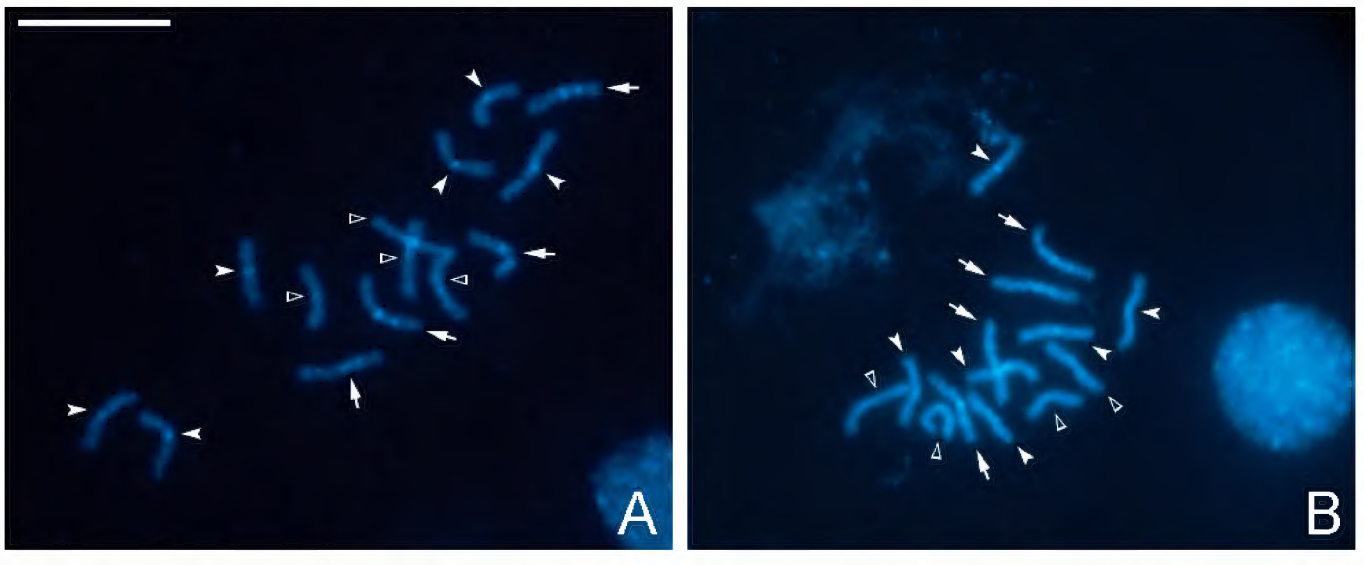

Figure 2. C-banded and DAPI-stained diploid metaphase plates of *P. dignus* (**A, B**). Arrows indicate chromosomes with both centromeric and interstitial C-positive bands, filled arrowheads indicate chromosomes with only centromeric C-positive bands, and empty arrowheads indicate lack of C-positive bands respectively. Scale bar: 10 μm.

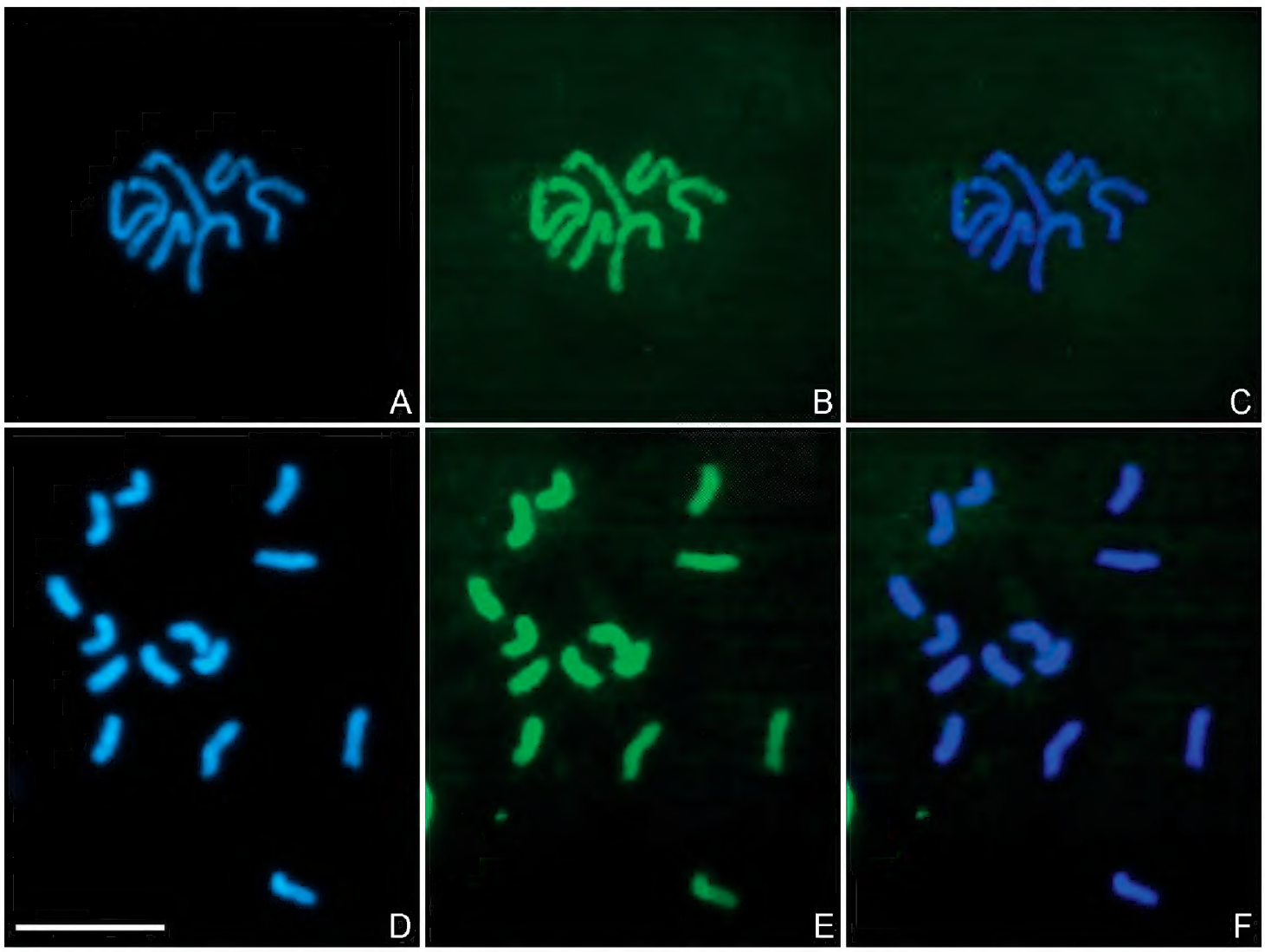

Figure 3. DAPI/CMA_3-stained metaphase haploid (**A–C**) and diploid (**D–F**) plates of *P. dignus* **A, D** DAPI staining **B, E** CMA_3 staining **C, F** merged images. Scale bar: 10 μm.

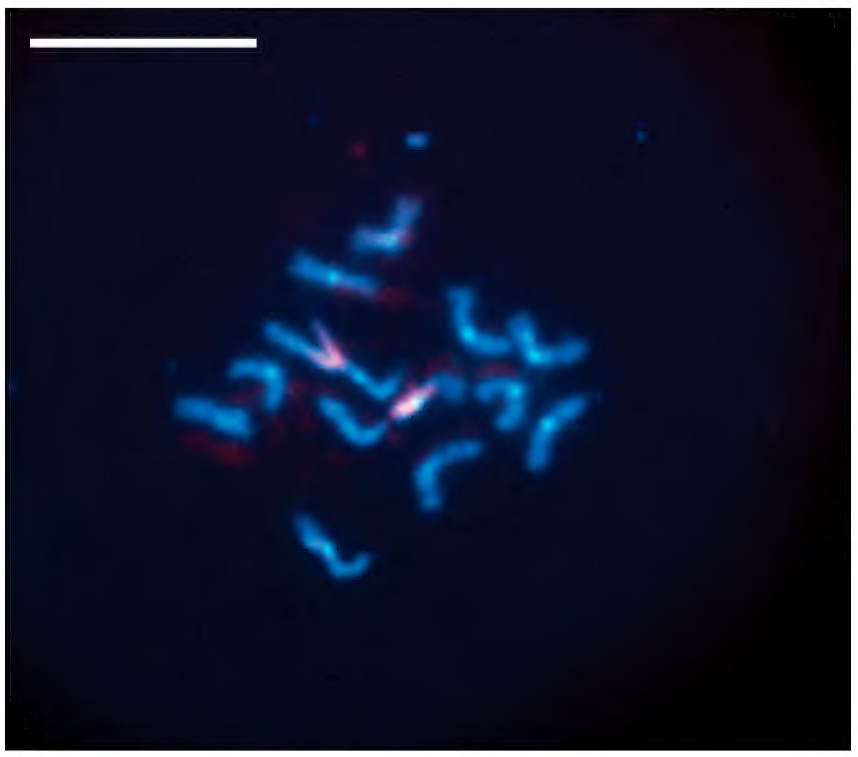

Figure 4. FISH with 18S rDNA probe on chromosomes of the diploid karyotype of *P. dignus*. Probe signals are indicated in red. Scale bar: 10 μm.

Discussion

The karyotype of *P. dignus* is characterized by the lowest chromosome number found in the subfamily Microgastrinae, with n values for other species ranging from 9 to 11 (Table 2). Moreover, members of this group exhibit considerable diversity in terms of chromosomal morphology. Specifically, karyotypes of most studied species of Microgastrinae, including *P. dignus*, *Cotesia glomerata* (Linnaeus, 1758) and *C. congregata*, predominantly contain biarmed chromosomes (Belle et al. 2002; Zhou et al. 2006). On the other hand, the chromosome set of "*Apanteles* sp." mostly harbors subtelocentric and/or acrocentric chromosomes (Hoshiba and Imai 1993). According to our phylogenetic reconstruction of karyotype evolution in Braconidae (Gokhman 2009), n values of 9 to 11 also prevail in the non-cyclostome lineage of this family; thus, the lower chromosome number, n = 7 found in *P. dignus*, is apparently derived. This lends further support from the suggested basal position of the genus *Microplitis* Förster, 1862 (Quicke 2015 and references therein), with n = 10–11 (Table 2).

Up to now, only three species of the family Braconidae have been studied using C-banding, "*Apanteles* sp." (Hoshiba and Imai 1993), *Aphidius ervi* Haliday, 1834 (n = 5, 2n = 10 and 12) (Gokhman and Westendorff 2003), and *Diachasmimorpha longicaudata* (Ashmead, 1905) (n = 20, 2n = 40) (Carabajal Paladino et al. 2013). In "*Apanteles* sp.", the karyotype consists of a single pseudoacrocentric chromosome with the fully heterochromatic shorter arm, along with nine more or less euchromatic subtelocentrics/acrocentrics together with an apparently euchromatic submetacentric chromosome (Hoshiba and Imai 1993). Chromosomes of *A. ervi* are also predominantly euchromatic; however, a few studied females carried an additional pair of almost fully heterochromatic acrocentric chromosomes (Gokhman and Westendorff 2003). In *D. longicaudata*, most chromosomes are pseudoacrocentric, and many of them carry large segments of pericentromeric heterochromatin (Carabajal Paladino et al. 2013). Our results obtained using C-banding in *P. dignus* thus revealed differences in the size and location of heterochromatic seg-

Table 2. Chromosome numbers of parasitoids of the subfamily Microgastrinae.

Species	n(2n)	Reference
Apanteles sp.[†]	11	Hoshiba and Imai 1993
Cotesia congregata (Say, 1836)	10	Belle et al. 2002
C. glomerata (Linnaeus, 1758)	10(20)	Zhou et al. 2006
Microgaster luctuosa Haliday, 1834 (= *curvicrus* Thomson, 1895)	(18)	Gokhman 2004
Microplitis demolitor Wilkinson, 1934	10	M. Strand, pers. comm., cited in: Gokhman 2009
M. ratzeburgii (Ruthe, 1858)	(22)	Gokhman 2009
M. tuberculifer (Wesmael, 1837)	(22)	Gokhman 2009
Pseudapanteles dignus (Muesebeck, 1938)	7(14)	Present paper

[†]Like many other identifications in the cited work, this one is dubious and may well refer to any other member of Microgastrinae, e.g., *Microplitis* sp.

ments compared to other Braconidae. Moreover, three different C-banding patterns were found within the diploid chromosome set of *P. dignus*, resulting in a species-specific distribution of constitutive heterochromatin. Various mechanisms have been proposed to account for the variation in the content and distribution of heterochromatin both within and between species, e.g., multiple replication, unequal exchanges, accumulation or elimination [reviewed by John (1988)], which could explain the differences observed among the four members of this family with known heterochromatin distribution.

Previously, multiple rDNA loci per haploid karyotype have been detected in certain Hymenoptera species using Ag-NOR, DAPI/CMA_3-banding and/or FISH; however, typically, only a single rDNA cluster is active (Hirai et al. 1994; Matsumoto et al. 2002; Gokhman 2009; Carabajal Paladino et al. 2013). In this order, as well as in other insects, CMA_3-positive bands co-localize with nucleolus organizing regions (NORs), suggesting that rDNA clusters are typically rich in GC base pairs (Camacho et al. 1991; Hirai et al. 1994; Vitturi et al. 1999; Maffei et al. 2001; Costa et al. 2004; Papeschi and Bressa 2006; Bolsheva et al. 2012; Gokhman et al. 2016; Gokhman and Kuznetsova 2024). However, in the parasitoid *D. longicaudata* (Carabajal Paladino 2011) and in certain true bugs (Hemiptera, Heteroptera) (Bressa et al. 2005; Severi-Aguiar and de Azeredo-Oliveira 2005; Morielle-Souza and Azeredo-Oliveira 2007; Poggio 2012), no such association has been demonstrated. The results of DAPI/CMA_3-banding indicate that all chromosomes of *P. dignus* lack specific regions enriched either in AT or GC base pairs. Thus, the NOR of *P. dignus* is apparently not associated with CMA_3-positive chromosomal segments as well.

To date, the only karyotypic study of Microgastrinae involving *in situ* hybridization was performed on *C. congregata* (Belle et al. 2002). Specifically, this technique visualized a single cluster of rDNA as well as certain DNA sequences coding for a symbiotic polydnavirus. In the haploid karyotype of this species, both sites appeared to have subterminal localization on shorter arms of the two different subtelocentric chromosomes (Belle et al. 2002). Contrary to *C. congregata*, all chromosomes of *P. dignus* are metacentric, but the single NOR is also located subterminally on a particular chromosome of the latter species. Interestingly, six rDNA clusters per haploid karyotype were earlier discovered in *D. longicaudata*, but, nevertheless, they all also have subterminal localization on chromosomes (Carabajal Paladino et al. 2013).

Most NORs in eukaryotic genomes are located in heterochromatic regions (Goessens 1984; Hadjiolov 1985; Babu and Verma 1987; Gokhman and Kuznetsova 2024), likely because certain heterochromatin-associated genes can silence repetitive DNA sequences and suppress recombination among them (Gottlieb and Esposito 1989). Based on the length and morphology of the chromosomes of *P. dignus*, we conclude that rDNA clusters in this species are located within the C-positive interstitial bands of one of the two chromosome pairs that carry these bands (Fig. 1B; see above). A similar pattern was previously observed in *D. longicaudata* (Carabajal Paladino et al. 2013). In this parasitoid species, hybridization signals with the 18S rDNA probe were also detected in heterochromatic regions. Nevertheless, both these regions and the rDNA clusters were CMA_3-negative and, consequently, not enriched in GC base pairs.

Although currently no karyotypically distinct groups of cryptic species of Microgastrinae are known, this situation may change as an increasing number of members

of this subfamily are examined, similarly to other taxa of parasitic wasps (Gokhman 2009, 2022). Moreover, chromosomal analysis of Microgastrinae will provide us further insights into their genetic features, which can, in turn, offer important information necessary for mass rearing and other aspects of applied use of these parasitoids. In addition, the results of the karyotypic study of this subfamily are already being used to verify the results of chromosome-level genome assemblies (Gokhman 2022, 2023). For example, this includes *C. congregata* and *C. glomerata* (Gauthier et al. 2021; Pinto et al. 2021). Furthermore, both genome assemblies of *Microplitis manilae* Ashmead, 1904 suggest n = 11 for this parasitoid (Shu et al. 2023; Yan et al. 2023). Since we report the same n value for two other *Microplitis* species (Table 2), these results appear plausible.

Author contributions

VEG: conceptualization, data curation, formal analysis, investigation, methodology, supervision, validation, visualization, writing – original draft, writing – review and editing. MGL: data curation, funding acquisition, methodology, resources, validation, writing – review and editing. CV: methodology, resources, writing – review and editing. MJB: conceptualization, data curation, formal analysis, investigation, methodology, resources, validation, visualization, writing – review and editing. All authors have read and agreed with the final version of the manuscript.

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